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3 **A DIVA system based on the detection of antibodies to non-structural**
4 **protein 3 (NS3) of Bluetongue virus**

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22

23

24 **Abstract**

25 Vaccination programs for the control of bluetongue (BT) in ruminants have limitations
26 due to difficulties in differentiating between vaccinated and virus infected animals
27 (DIVA). To overcome this problem a DIVA test that looks at a differential immune
28 response to bluetongue virus (BTV) non-structural protein 3 (NS3) was developed. The
29 NS3 encoding gene of strain BTV4/22045/PT04 was inserted into expression vector
30 pET-28a and expressed in *Escherichia coli* strain JM109. Recombinant NS3 protein
31 was used as an antigen in an indirect ELISA (NS3-ELISA) to measure the serologic
32 response to NS3 protein in cattle and sheep. Following a cattle vaccination/challenge
33 experiment with a bivalent inactivated BTV 2-4 vaccine, NS3 antibodies were detected
34 at approximately 15 days post-infection in control unvaccinated animal, while
35 vaccinated animals did not develop detectable NS3 antibodies and, with exception of
36 one, remained negative even after virus challenge. The inactivated vaccine induced
37 antibodies against the major core structural protein (VP7) of BTV as well as
38 neutralizing antibodies in all animals.

39 To evaluate the applicability of NS3-ELISA in field scenario, a total of 562 serum
40 samples collected from uninfected, BTV infected and vaccinated animals were tested
41 for NS3 antibodies. Taken together, the results confirm that NS3 antibodies were
42 induced to the greatest levels in animals infected with BTV in comparison to the levels
43 induced in animals immunized with inactivated BTV vaccines, implying that antibody
44 response to NS3 allows the differentiation between infected and vaccinated animals.

45

46 *Keywords:* Bluetongue virus; Non-structural proteins; NS3; ELISA; DIVA

47 **Introduction**

48 Bluetongue is a vector-borne viral disease of ruminants, including sheep, goats and
49 cattle, caused by bluetongue virus (BTV). Clinical signs are seen mainly in sheep but
50 recently clinical cases have been also observed in cattle (Toussaint et al., 2006). BTV is
51 a member of the *Orbivirus* genus within the family *Reoviridae*, possessing 10 double-
52 stranded RNA segments enclosed by three consecutive capsid layers of multiple
53 proteins. In addition to the seven structural (VP1 to VP7), there are three non-structural
54 (NS1, NS2 and NS3) proteins. The smallest RNA segment (S10) of BTV encodes two
55 closely related proteins, the NS3 (25.5 kDa) and NS3A (24 kDa), which are read from
56 alternative in frame methionine initiation codons (Roy et al., 1990). The NS3A
57 expression is more variable and generally occurs at a much lower level than that of NS3
58 (Wu et al., 1992). The NS3 protein is essential for both virus assembly and release from
59 infected cells (Bansal et al., 1998; Hyatt et al., 1993; Wirblich et al., 2006).

60 Since 1998, seven BTV serotypes (BTV-1, 2, 4, 6, 8, 9 and 16) have invaded Europe
61 (Maan et al., 2007; Toussaint et al., 2006; Ref OIE: 7458/October/2008). In Portugal the
62 first outbreak of BTV-4 occurred in 2004 (Barros et al., 2007) in the South of the
63 country. In 2007, a new incursion of BTV, this time of serotype 1, was reported (Ref
64 OIE: 6248/September/2007) and since then the virus has spread across the country.

65 Vaccination is the most effective mean to protect animals against disease and to reduce
66 the spread of virus after infection. Unfortunately, vaccination interferes with the
67 serological detection of infected animals. Inactivated vaccines against serotypes 1, 2, 4,
68 and 8 of BTV were recently developed and have been used in the European Union,
69 replacing the conventional live attenuated vaccines. In Portugal, a bivalent inactivated
70 vaccine against serotypes 2 and 4 was firstly used in 2005 but was subsequently
71 superseded by monovalent vaccines for BTV-4 and BTV-1. These vaccines offer

72 significant advantages over attenuated vaccines because of the absence of replicating
73 virus.

74 The non-structural NS3 protein is produced in large quantities in infected cells, but is
75 not packaged in the virion. Since inactivated vaccines for BTV are primarily made with
76 whole virions, a differential antibody response should be observed between naturally
77 infected and vaccinated animals. Therefore, NS3 antibody response is related *in vivo* to
78 the virus activity.

79 For both trade and surveillance purposes, it is important not only to differentiate
80 naturally infected and vaccinated ruminants, but also to identify vaccinated animals that
81 become infected with BTV.

82 The main goal of the present study was to establish a diagnostic test for differentiation
83 between vaccinated and BTV infected animals. We describe the development of an
84 indirect ELISA with a DIVA function that uses a recombinant non-structural NS3
85 protein as an antigen for the detection of BTV-NS3 antibodies in cattle and sheep. This
86 NS3-ELISA was evaluated with sera collected from naive, vaccinated and infected
87 animals and the performance of the method was compared with a commercial
88 competitive ELISA (cELISA), serum neutralization test (SN) and with RT-PCR to
89 determine BTV infection status.

90

91 **Materials and Methods**

92 **2.1. Expression and purification of recombinant NS3 protein**

93 The DNA fragment encoding NS3, from BTV4/22045/PT04 (GenBank accession
94 number EF434180), contained in a pCR2.1 vector (Invitrogen) was amplified by using
95 the primers: *NheI*-NS3-F (5'-GCTAGCATGCTATCCGGGCTGAT-3') and *EcoRI*-
96 NS3-R (5'-GAATTCTCGGTTAATGGTAATTCG-3') inserting *NheI* and *EcoRI*

97 cleavage sites. PCR was undertaken for 40 cycles (30 s at 94 °C; 30 s at 55 °C, 1 min 20
98 s at 72 °C) with a final extension at 72 °C for 5 min. Amplified fragments were digested
99 with *NheI* and *EcoRI* and cloned in the expression vector pET-28a(+) (Novagen) in-
100 frame with amino and carboxy terminals 6xHis-tag epitopes. The recombinant plasmid
101 pET28a/NS3 was then transformed into *E. coli* JM109. Cells were grown at 37 °C in LB
102 medium containing 25 µg/ml of kanamycin, and protein expression induced with 1 mM
103 IPTG when the cell density (OD₆₀₀) reached 0.6. The cultivation was continued for 4 h
104 and cells were harvested by centrifugation at 5000 x g for 10 min at 4 °C.
105 Recombinant NS3 was solubilized from inclusion bodies as previously described (Lu et
106 al., 2007), with minor modifications. The concentrated bacterial pellets were washed
107 twice in 0.25 x culture volume buffer (20 mM Tris-HCl, pH 7.5) and resuspended in
108 inclusion body (IB) lysis buffer (20 mM Tris-HCl, pH 7.5; 10 mM EDTA; 1% Triton
109 X-100) supplemented with lysozyme to a final concentration of 100 µg/ml. The mixture
110 was incubated at room temperature for 15 min, sonicated on ice until the solution was
111 no longer viscous, and the inclusion bodies were recovered by centrifugation. After two
112 additional washes in lysis buffer, the inclusion bodies were solubilized by the addition
113 of IB solubilization buffer (50 mM CAPS, pH 11; 0.3% N-lauroylsarcosine; 1 mM
114 DTT). The supernatant containing the solubilized protein was collected for subsequent
115 binding to NI-NTA His binding resin (Qiagen), according to manufacture's instructions.
116 The amount of total proteins was determined by Pierce BCA kit.

117

118 **2.2. Indirect ELISA procedure for detection of NS3 antibodies (NS3-ELISA)**

119 The optimal concentration of antigen and dilution of the serum were determined by
120 performing a checkerboard titration of the NS3 antigen and positive and negative bovine
121 and ovine control sera. Based on this initial titration, 150 ng/ 100µl of antigen and 1:10

122 serum dilution were used in all further NS3-ELISA. Microtiter plates were coated with
123 NS3 antigen diluted in carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at
124 4 °C. Following 4 washes with PBS/0.05% Tween-20, serum samples diluted 1:10 in
125 dilution buffer (PBS containing 5% skimmed milk and 3% *E.coli* lysate), were added at
126 100 µl/well and incubated for 30 min at room temperature. Plates were washed and
127 donkey anti-sheep/goat-IgG-HRP (1:20000) or sheep anti-bovine-IgG-HRP conjugate
128 (1:1500) both from Serotec, was added, 100 µl/well, in the same dilution buffer and
129 incubated for more 30 min at room temperature. After four wash cycles, colour was
130 developed with 100 µl TMB reagent (Sigma) and the reaction was allowed to proceed
131 for 15 min before being stopped by adding 0.5 M H₂SO₄. The optical density (OD) was
132 read at 450 nm using a microplate reader (Tecan). The samples and positive control OD
133 values (OD_{sample} and OD_{pos}) were corrected by subtracting the OD value of the negative
134 control (OD_{neg}): Percentage Positivity (PP) = [(OD_{sample} - OD_{neg}) / (OD_{pos} - OD_{neg})] x
135 100%. Samples with a PP ≥40% were considered positive, between 35-40% were
136 considered inconclusive and those <35% were negative. The test was considered valid if
137 OD_{pos} / OD_{neg}>5.

138

139 2.3. Source of sera

140 A group of 7 bovines (numbered, N1 to N7) maintained in vector-free premises were
141 immunised with a bivalent inactivated vaccine (BTVPUR AlSap 2-4, Merial). Two
142 subcutaneous immunizations were performed with 3 weeks apart. Sixty days after the
143 first immunization the 7 vaccinated and one control animal (N8) were challenged
144 intravenously with strain BTV4/22045/PT04 ($6.5 \pm 1.1 \times 10^{-5}$ PFU/5ml). Blood samples
145 collected before and after challenge were tested by NS3-ELISA.

146 A total of 562 field samples of non-vaccinated animals from BTV-free areas, from
147 vaccinated animals with BTVPUR AlSap 4 (Merial) and/or BTV-1 Zulvac 1 (Fort
148 Dodge), as well as samples from infected animals collected during BTV-1 and BTV-4
149 outbreaks in 2007 and 2008, were studied.

150

151 **2.4. Commercial ELISA test**

152 Bluetongue virus antibody test kit (VMRD, *Inc.*, Pullman, WA, USA), a competitive
153 ELISA (cELISA) was used as a standard for comparison. The test is based on the
154 detection of antibodies specific to the highly conserved VP7 protein of BTV.

155

156 **2.5. Serum neutralization test (SN)**

157 Samples were tested for the presence of neutralizing antibodies. Serial dilutions (1:10)
158 of heat-inactivated sera were incubated for 1h at 37 °C with an equal volume (50 µl) of
159 BTV (100 TCID₅₀), in a 96-well microplate. Approximately 10⁴ BHK₂₁ cells in 100 µl
160 of medium were added per well and after incubation for 5 days at 37 °C with 5% CO₂,
161 the microplate was read using an inverted microscope. Two replicates of each serum
162 were tested with BTV-1 (BTV1/PT/29058/07), BTV-2 (BTV2/26629/PT05) or BTV-4
163 (BTV4/22045/PT/05). Neutralizing antibody titers were calculated according to
164 Spearman-Kärber's formula and expressed as the last dilution of serum that neutralizes
165 CPE in 100%. Serum samples with a virus neutralisation titer of $\geq 1.3 \log_{10}$ were
166 considered positive.

167

168 **2.6. Real time RT-PCR (rRT-PCR)**

169 The Pan BTV (NS1 based) rRT-PCR was routinely used as reference test to determine
170 the true status of BTV infection in animals, as previously described (Barros et al.,

171 2007). BTV serotyping was carried out by rRT-PCR using specific primers for BTV-1
172 VP2 (155F-5'-GAATGCATATGACATCAAGCAG-3'; 279R-5'-
173 CGTCTTTCATCGTAACCCC-3'; probe-5'FAM-
174 ACCGCATTATGGTATAACCCGATAAGA-3'TAMRA) and BTV-4 VP2 (F-5'-
175 ATTATACGCTTGGGAAGTAC-3'; R-5'-AATTAGTCAATGCAGGGAAG-3';
176 probe-5'FAM-TTTGAGTCAGAAACAAACCCAATCAGCCGT-3'TAMRA).

177

178 **3. Results**

179 **3.1. Generation of recombinant NS3 protein for development of an indirect ELISA** 180 **(NS3-ELISA)**

181 In order to investigate the applicability of NS3 as DIVA test, the NS3 gene of
182 BTV4/22045/PT04 isolate was cloned in vector pET-28a, creating a His-tagged
183 recombinant NS3 protein, confirmed in the Western blot analysis by anti-His-tag
184 monoclonal antibody as well as by BTV positive serum (Fig. 1). The expressed protein
185 was used as antigen for developing an indirect ELISA (NS3-ELISA).

186

187 **3.2. Evaluation of NS3-ELISA with sera from vaccination trial**

188 **3.2.1. Serological results**

189 Sera from seven bovines that participated in a vaccination/challenge experiment with
190 the inactivated vaccine BTVPUR AlSap 2-4 (Merial) were tested in NS3-ELISA, c-
191 ELISA and SN assay. The animals were given one dose of vaccine subcutaneously, and
192 3 weeks later a booster immunisation was performed. Sixty days after the primo-
193 vaccination, all the seven immunized animals and one unvaccinated control animal (N8)
194 were challenged with virus strain BTV4/22045/PT04. Before vaccination all animals
195 were serologically and virologically negative for BTV.

196 As shown in Figure 2, none of the immunized animals developed significant antibodies
197 levels against NS3 antigen before challenge, indicating that bivalent vaccine did not
198 induce NS3 antibodies. In contrast, antibodies against BTV structural protein VP7 were
199 detected by cELISA at day 21 post-vaccination in three of the seven immunized animals
200 and 7 days later the four remaining animals also seroconverted. All these animals
201 maintained detectable levels of VP7 antibodies until the end of the study (216 days).
202 Neutralizing antibodies were first detected 14 days (BTV-2) and 21 days (BTV-4) post-
203 vaccination and by days 21 and 28 all animals had developed BTV-2 and BTV-4
204 neutralizing antibodies, respectively. The mean neutralizing antibody titers peaked on
205 day 14 after boosting (2,4 log₁₀ for BTV-2 and 2,0 log₁₀ for BTV-4) and raised up to a
206 mean of 2,7 log₁₀ after challenge with BTV-4, being detectable until day 216 when the
207 assay ended (Fig. 3).
208 Following challenge, all vaccinated animals remained NS3 seronegative with the
209 exception of animal N7 that developed a weak and short lasting (~15 days) antibody
210 response to NS3 (Fig. 2).
211 Before challenge the unvaccinated control animal (N8) was serological and virological
212 negative for BTV. After inoculation with BTV4/22045/PT04 the NS3 antibody levels
213 started to rise between 8-15 day post-infection (pi) reaching a maximum at
214 approximately 43 pi and lasted over 141 days (Fig. 2). By using the cELISA, antibodies
215 against VP7 were detected approximately 8 days pi, a week earlier than NS3 antibodies.
216 Neutralizing antibodies to BTV-4 were also detected after inoculation, peaking around
217 60 days pi (Fig. 3).
218 The exact duration of NS3 antibodies in the control animal (N8) was not determined.
219 Luckily an extra blood sample from this animal was received in the laboratory at day
220 330 pi and tested NS3-ELISA negative but VP7 cELISA positive.

221

222 3.2.2. rRT-PCR results

223 Blood samples were analysed for the presence of viral RNA by rRT-PCR (Fig. 4). Of
224 the seven immunised animals, two (N3 and N6) did not yield any positive rRT-PCR,
225 three (N1, N4 and N5) were positive between 1-3 days and one (N7) was positive up to
226 7 days post-challenge (pc). In this animal a low level replication of the challenging virus
227 may have occurred since a low titer and short lasting NS3 antibodies were detected (Fig.
228 2). On the other hand, the detection of viral RNA during the first three days pc in some
229 of the other animals may reflect circulation of the inoculums rather than viral
230 replication, since NS3 antibodies were not detected.

231 In control animal (N8), BTV-4 nucleic acid was first detected at 24h pi and was
232 continuously detected until the end of the trial (156 days pi). As inferred by the Ct
233 values of rRT-PCR, viral load steadily increased from day 12 up to day 20 pi, and then
234 began to decrease (Fig. 4). The rRT-PCR was able to give a positive signal up to 15
235 days before antibodies could be detected by NS3-ELISA while the delay time for
236 cELISA was 8 days. As referred above, an extra blood sample from animal N8 was
237 obtained at day 330 pi. Interestingly enough, in this sample the challenge virus could
238 still be detected by rRT-PCR (Ct value = 36.8) while NS3-specific antibodies were no
239 longer detectable.

240

241 3.3. Field application study of NS3-ELISA

242 For further validation of the NS3-ELISA as a DIVA test, a total of 562 field serum
243 samples originated from uninfected, vaccinated or naturally infected animals were used.
244 These samples were also tested by cELISA, SN assay and rRT-PCR.

245 The mean and SD of the OD of the sera obtained from uninfected animals were $0.11 \pm$
246 0.07 and 0.13 ± 0.05 in ovine and bovine, respectively. For vaccinated sheep and cattle,
247 the mean and SD of OD values were respectively 0.17 ± 0.08 and 0.17 ± 0.07 (Table 1).
248 A good discrimination between positive and negative samples was observed; the
249 difference between the average OD value of samples from infected (NS3 positive) and
250 vaccinated (VP7 positive/ NS3 negative) animals was 0.84 and 0.73, respectively for
251 sheep and cattle.

252 To evaluate the ability to diagnose infected animals the NS3-ELISA was compared with
253 rRT-PCR (Table 2). The correlation between NS3-ELISA and rRT-PCR was 87% and
254 51% respectively for sheep and bovine positive samples, while for negative samples the
255 correlation was respectively 98% and 100%. All discrepant samples were analysed at
256 least three times.

257 Of 135 ovine BTV-1 rRT-PCR positive samples, 17 (13%) were negative by NS3-
258 ELISA. Nine of these discrepant samples were also negative in cELISA and SN test,
259 suggesting a very early infection prior to seroconversion. The remaining eight samples,
260 positive for cELISA, originated from vaccinated animals with neutralizing antibodies
261 for BTV-4 but not for BTV-1, suggesting a recent infection with BTV-1 (Table 3).

262 From a total of 293 rRT-PCR negatives samples, only three from sheep with clinical
263 signs were positive by NS3-ELISA and cELISA.

264 Sixty-seven discrepant rRT-PCR positive and NS3-ELISA negative bovine samples
265 were recorded (49%). These animals were separated into two groups, BTV-1 (24
266 animals) or BTV-4 (43 animals) rRT-PCR positive. The samples from the latter group
267 were collected between December 2007 and February 2008, when vector activity was
268 reduced or absent and outbreaks of BT were not registered. Two animals of this group
269 were bled twice with one month interval and the second collection resulted rRT-PCR

270 negative, indicating that viral RNA can be detected for a longer period than NS3
271 antibodies. As previously determined for bovine N8, these animals could be in a late
272 stage of infection.

273 In the group of 24 BTV-1 positive cattle, originated from farms located in areas where
274 BTV-1 outbreaks were occurring, two animals were NS3 and VP7 negative, suggesting
275 a very early stage of infection, while the remaining samples, NS3 negative and VP7
276 positive, are likely to originate from recent infected animals that seroconverted to VP7
277 but not yet to NS3. In one case it was possible to demonstrate seroconversion to NS3
278 from paired samples received with one week interval.

279

280 **3.4. Inter- and intra-assay variations of NS3-ELISA**

281 To calculate the inter-assay variation (reproducibility) of NS3-ELISA, the OD values of
282 a pool of positive sera was measured 6 times on different days. The coefficient of
283 variation for ovine sera was 0.067 and for bovine sera was 0.028.

284 For intra-assay variation (variability), OD values of six replicates of the same serum
285 were measured in six columns of the plate. The coefficient variation was 0.016 and
286 0.022 respectively for ovine and bovine sera.

287

288 **Discussion**

289 In the present work an indirect ELISA was developed for detection of BTV antibodies
290 against non-structural NS3 protein, which enables the differentiation between infected
291 and vaccinated animals (DIVA). This method is theoretically based on the assumption
292 that inactivated BTV-vaccines mainly contain capsid proteins, so they are less likely to
293 illicit production of antibodies against non-structural proteins.

294 Following a vaccination/challenge experiment, with a bivalent inactivated BTV2-4
295 vaccine, seven vaccinated and one unvaccinated control bovine were evaluated for the
296 ability to develop antibodies to NS3. All vaccinated animals were negative by NS3-
297 ELISA and, with exception of one (N7), remained negative even after challenge. In
298 animal N7 a low level replication of the challenging virus may have occurred since viral
299 RNA was detected up to 7th day pc. This brief viraemia could be responsible for the
300 low titer and short lasting NS3 antibodies observed after challenge. In contrast, all
301 vaccinated animals developed antibodies against BTV, as measured by commercial
302 cELISA and SN test. These animals were protected since viraemia was no longer
303 detected by rRT-PCR soon after challenging virus inoculum was cleared from blood
304 stream (3 days pc). The efficacy of this inactivated vaccine was also demonstrated in a
305 previous study (Savini et al., 2009).

306 The unvaccinated control animal (N8) developed NS3 and VP7 antibodies in response
307 to challenge infection. Concerning the antibodies appearance, VP7 antibodies were
308 detected in the serum 8 days pc, whereas NS3 antibodies appear about one week later.
309 The exact duration of NS3 antibodies could not be determined, but they were no longer
310 detected on day 330 pc, while VP7 antibodies were still detectable. Surprisingly, this
311 sample was rRT-PCR positive for challenging virus on this date. Therefore, if viral
312 replication ever occurred in the control animal between 216-330 days pc, it was not
313 enough to stimulate NS3 antibodies at detectable levels.

314 The dynamic of the appearance of NS3 antibodies were also studied in another trial
315 involving five BTV-4 immunized sheep with inactivated vaccine BTVPUR AlSap 4
316 (Merial). These animals were NS3 antibody negative and VP7 antibody positive. After
317 inoculation with BTV-1 (BTV1/PT/29058/07) the animals seroconvert to NS3 on day
318 10 pi (data not shown). As for the bovine trial described above, this experiment with

319 sheep also showed the ability of NS3-ELISA to differentiate between infected and
320 vaccinated animals.

321 Previous studies demonstrate that viraemia in sheep and cattle could be prolonged up to
322 38-54 days as determined by virus isolation (Koumbati et al. 1999; Bonneau et al.,
323 2002). However, BTV nucleic acids can be still detected by RT-PCR on a longer period,
324 up to 180 and 222 days pi in cattle and sheep, even if it does not mean that infectious
325 particles are present (Katz et al., 1994; Bonneau et al., 2002). The RT-PCR assay
326 potentially would detect viral RNA contained in any inactivated virions or subviral
327 particles bound to circulating erythrocytes or other blood cells (Brewer and
328 MacLachlan, 1992). Thus, ruminants whose blood contains RT-PCR detectable BTV
329 nucleic acid, but not infectious virus as determined by virus isolation, likely are
330 unimportant to the epidemiology of BTV infection (MacLachlan et al., 1994;
331 Tabachnick et al., 1996; Bonneau et al., 2002).

332 To further evaluate the applicability of NS3-ELISA as a DIVA test, we analysed a
333 larger number of field samples from infected, vaccinated, and from virological and
334 serological negative animals of BTV free areas. Once again the results show that BTV
335 infected animals develop higher levels of NS3 antibodies compared with the response
336 elicited by inactivated vaccines, which induced little or no detectable antibodies to NS3
337 protein. The mean OD value of NS3-ELISA for sera from vaccinated animals was
338 almost identical to that obtained with sera from BTV negative animals, allowing a good
339 discrimination between positive and vaccinated animals.

340 Although the RT-PCR does not detect the same biological entity as the ELISA (viral
341 nucleic acid versus antibodies), we compare both results in order to evaluate the ability
342 of NS3-ELISA to diagnose infected animals. Between the two tests, a concordance of
343 87% was found for sheep positive samples. Half of the sheep discrepant samples, BTV-

344 1 rRT-PCR positive but NS3-ELISA negative were also negative for cELISA,
345 suggesting a very early infection (NS3, VP7, and SN negative/rRT-PCR positive). The
346 remaining discrepant samples (rRT-PCR positive/NS3-ELISA negative) were cELISA
347 positive and originated from vaccinated animals with neutralizing antibodies for BTV-4
348 but not for BTV-1, suggesting, an early BTV-1 infection (NS3 negative, VP7 positive,
349 and SN negative/rRT-PCR positive). VP7 antibodies can be present due to previous
350 BTV-4 immunization or due to a recent BTV-1 infection in which the faster antibody
351 response to VP7 is already present but antibodies to NS3 are not yet detectable. In the
352 vaccination trial, NS3 antibodies appeared one week later than those to VP7 in the
353 control unvaccinated animal after challenge.

354 Considering the bovine samples, the percentage of discrepancies (rRT-PCR
355 positive/NS3-ELISA negative) was 49% and it can be classified in two types. As in
356 sheep, one type of samples may be originated from animals in very early or early stages
357 of infection. At least in one case, this assumption was confirmed with paired samples
358 collected with a week interval in which seroconversion to NS3 was observed.
359 Moreover, these animals were located in the vicinity of sheep farms where outbreaks of
360 BTV-1 were occurring.

361 The other type of discrepancy was observed in samples with an rRT-PCR positive result
362 for BTV-4 in which BTV-4 neutralizing antibodies were detected but not NS3
363 antibodies. It has been demonstrated before that appearance of neutralizing antibodies in
364 serum does not coincide with clearance of virus from blood and BTV may coexist with
365 specific neutralizing antibodies in infected animal for long periods (Richards et al.,
366 1988). These samples were collected between December 2007 and February 2008, when
367 vector activity was reduced or absent and outbreaks were not registered. Two animals of
368 this group were bled twice with one-month interval and in second collection of blood

369 viral RNA was no longer detected. These results together with the high Ct values of
370 rRT-PCR, presence of BTV-4 neutralizing antibodies, winter time, and the absence of
371 new BTV-4 outbreaks reported during 2008, suggest that these samples originated from
372 animals in a late stage of infection (NS3 negative, VP7 positive, SN-BTV-4 positive
373 and rRT-PCR-BTV-4 positive). As observed for control animal (N8), viral RNA could
374 be detected for a longer period than NS3 antibodies.

375 Taking into account the epidemiological data (i.e., vector activity; the season;
376 occurrence of outbreaks; trade of animals; introduction of new serotypes), the use of
377 NS3-ELISA may be advantageous allowing the movement of positive rRT-PCR
378 animals in late stages of infection when viral RNA is still detected but NS3 antibodies
379 are no longer present.

380 A good agreement between rRT-PCR and NS3-ELISA for negative samples was
381 observed. From a panel of 293 rRT-PCR negative samples only three from sheep with
382 clinical signs of BT were NS3-ELISA positive. These discordant results are difficult to
383 explain, however, since NS3-ELISA and cELISA were concordant, it seems that
384 limiting factors of rRT-PCR, namely poor quality of RNA, RT-PCR inhibitors,
385 mutations that hamper primer or probe annealing or viral load below the limit of
386 detection, can be responsible for the negative detection of viral RNA. However, these
387 samples were several times re-analysed giving consistent results. Although maternal
388 antibodies could be another possible explanation, the age of the animals, over one year,
389 excludes this hypothesis. On the other hand, animals in which inactivated vaccines have
390 been repeatedly used may develop NS3 antibodies, due to possible presence of small
391 amounts of this protein in the vaccine. However, in the present study this hypothesis
392 was not confirmed with the vaccines used, since many of the NS3 antibody negative
393 field samples originated from BTV-1 or/and BTV-4 vaccinated animals that had been

394 revaccinated every six months. Finally, a short lasting viraemia could also be an
395 explanation as observed in animal N7 of the vaccination trial.

396 The purity status of the inactivated vaccines was a critical point since low levels of
397 antibodies against non-structural proteins can be produce. The inactivated vaccines used
398 in this study were sufficiently purified since it did not induce NS3 antibodies at levels
399 detectable by NS3-ELISA. However, tests with different vaccines from other companies
400 and for different BTV serotypes are needed to ascertain whether those vaccines induce
401 antibodies against NS3 protein. In a recent inter-laboratory proficiency test carried out
402 by the EU Community Reference Laboratory for BT (Institute for Animal Health,
403 Pirbright, UK), the NS3-ELISA did not detect NS3 antibodies in four samples from
404 cattle vaccinated with the 4 available BTV-8 inactivated vaccines (CZ Veterinaria, Fort
405 Dodge, Intervet and Merial). These preliminary results suggest that NS3-ELISA is not
406 serotype dependent.

407 Non-structural proteins have also been used in DIVA tests, for African horse sickness
408 virus (Laviada et al., 1995), foot-and-mouth disease virus (Clavijo et al., 2004; Lu et al.,
409 2007) and equine influenza virus infections (Birch-Machin et al., 1997). Also a
410 competitive ELISA based on the use of a monoclonal antibody directed against the NS1
411 protein of BTV allowing the discrimination between infected and vaccinated animals
412 has been described (Anderson et al., 1993).

413 The diagnostic test described in this study demonstrates that NS3 protein of BTV can be
414 used as a diagnostic reagent in DIVA tests. Although NS3-ELISA is less sensitive than
415 rRT-PCR detecting earlier infections, it's a method comparatively easy and cheap for a
416 routine diagnosis. Because of the imposed restriction to animal movement between
417 BTV-infected and non-infected areas, the NS3-ELISA can be used as a reliable tool for
418 massively serological survey to detect incursions of BTV into the animal population,

419 regardless of the serotype of the virus involved, while it would correctly exclude
420 animals that have been vaccinated.

421

422

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427

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Table 1: OD values of NS3-ELISA of 478 field sera from uninfected, infected and vaccinated sheep and cattle.

NS3-ELISA (OD _{450nm})	Sheep			Cattle		
	Uninfected ^a	Vaccinated ^b	Infected ^c	Uninfected ^a	Vaccinated ^b	Infected ^c
Mean (M)	0.12	0.18	1.00	0.13	0.17	0.88
Standard deviation (SD)	0.07	0.08	0.28	0.06	0.07	0.25
Total of animals	71	63	118	68	88	70

^a Uninfected animals: rRT-PCR (-), NS3-ELISA (-) and cELISA (-); ^b Vaccinated animals: rRT-PCR (-), NS3-ELISA (-), cELISA (+) and SN (+); ^c Infected animals: rRT-PCR (+), NS3-ELISA (+), cELISA (+).

Table 2. Comparison between the NS3-ELISA and the rRT-PCR using 562 field serum samples

NS3-ELISA	RT-PCR					
	Ovine			Bovine		
	Positive	Negative	Total	Positive	Negative	Total
Positive	118	3	121	70	0	70
Negative	17	134	151	67	156	223
Total	135	137	272	137	156	293

Table 3. Summary and interpretation of virological and serological results with 562 field serum samples

Number of sera		Results	Interpretation
sheep	cattle		
118	70	NS3(+)/VP7(+)/RT-PCR(+)	Infected
3	0	NS3(+)/VP7(+)/RT-PCR(-)	Resolved short lasting viraemia/previous infection / maternal antibodies / repeated vaccinations?
63	88	NS3(-)/VP7(+)/RT-PCR(-)	Vaccinated (inactivated vaccine)/previous infection
9	3	NS3(-)/VP7(-)/RT-PCR(+)	Very early infection
8	64	NS3(-)/VP7(+)/RT-PCR(+)	Early infection if SN (-)/Late infection if SN (+)
71	68	NS3(-)/VP7(-)/RT-PCR(-)	Uninfected

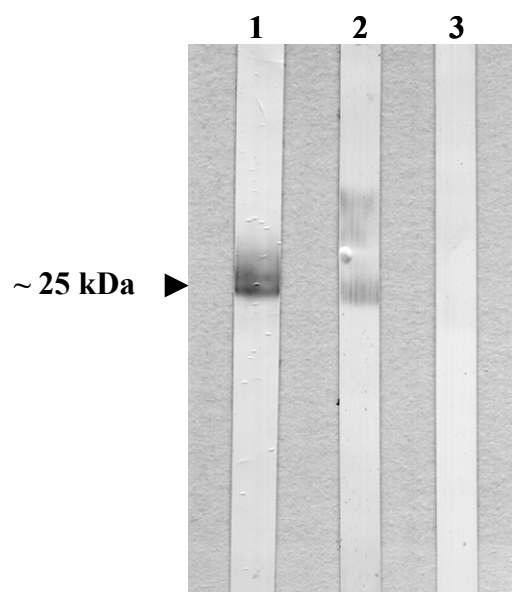


Figure 1: Western blot analysis of the NS3 immune response. Western blots developed with HRP-conjugated anti-His-tag monoclonal antibody (lane 1), of serum from BTV-1 infected sheep (lane 2) and of serum from uninfected sheep (lane 3).

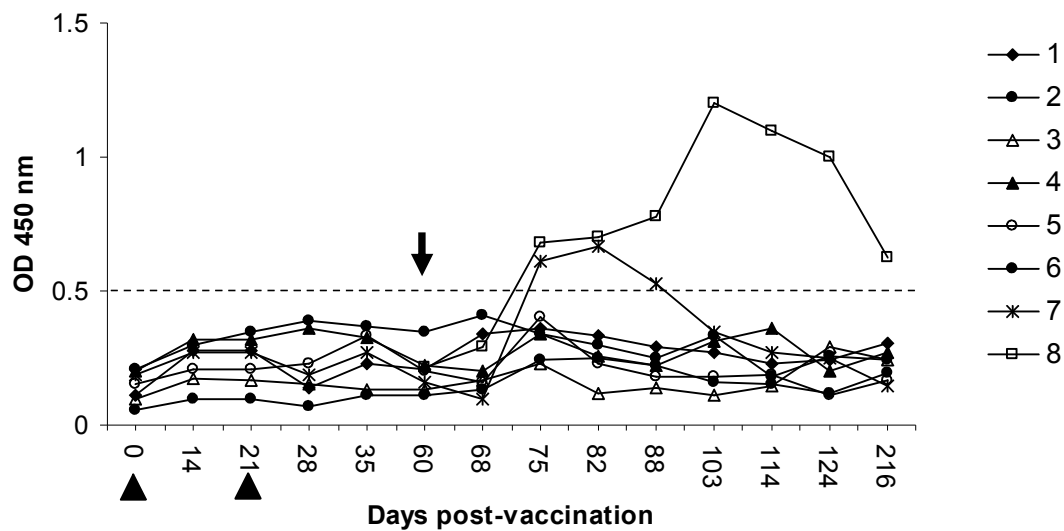


Figure 2: BTV-NS3 antibody response of animals included in the vaccination trial. Samples with an $OD_{450} \geq 0.5$ were considered positive. ▲ represent vaccine administration and the ↓ indicates the challenging day with BTV4/22045/PT04.

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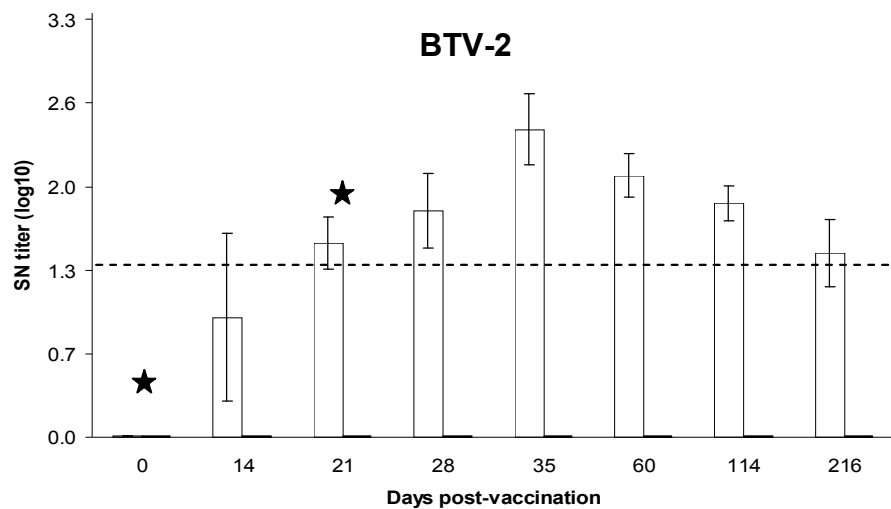
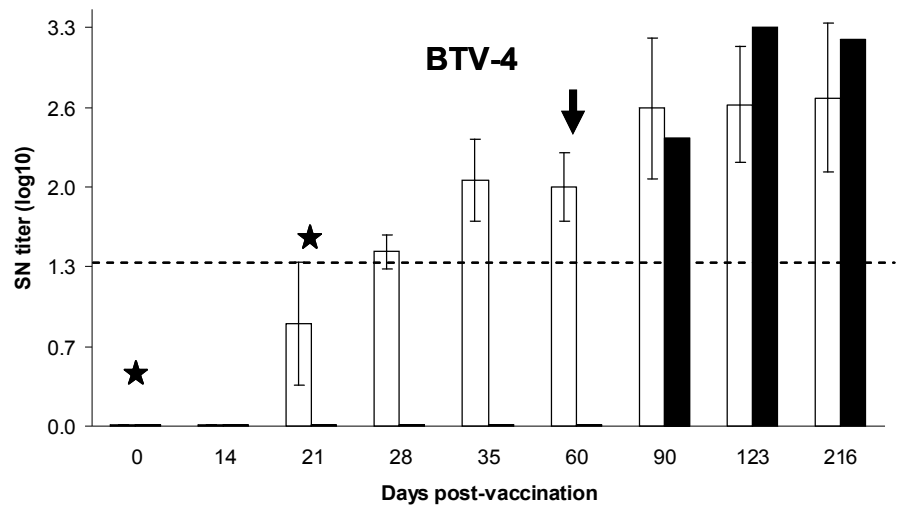


Figure 3. Mean titers (\pm S.D.) of BTV-2 and BTV-4 neutralizing antibodies of seven animals after immunization with BTVPUR AISap 2-4 inactivated vaccine. Control animal N8 is represented by a solid bar. The positive threshold is indicated by a dashed line (1.3 log₁₀). The vaccine administration is indicated by a ★ and the ↓ indicates the challenging day with BTV4/22045/PT04.

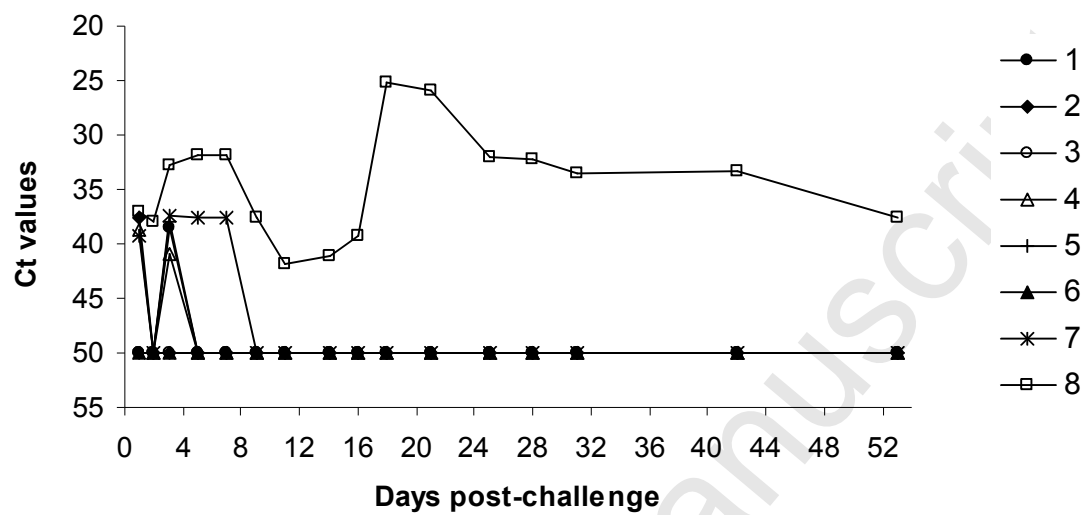


Figure 4. Detection of BTV viral RNA by rRT-PCR in control and vaccinated animals after challenge infection with BTV4/22045/PT04. For negative samples a threshold value of Ct=50 was used.